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28-02-2014	Final Report	4-Sep-2009 - 3-Sep-2013

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Final Report: Implementing Material Surfaces with an Adhesive Switch	W911NF-09-1-0479
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	5c. PROGRAM ELEMENT NUMBER 611102
6. AUTHORS Valeria Tohver Milam	5d. PROJECT NUMBER
	5e. TASK NUMBER
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13. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

The work focuses on implementing material surfaces that present temporal adhesive cues. We are exploring conformational changes in surface-immobilized oligonucleotides to promote an “off-on” switch in adhesion events on the surface of colloidal particles. The overall objective is to optimize oligonucleotide characteristics (e.g. base length, modified nucleotide content, etc.) for chemically-robust macromolecules in order to tailor both the timing as well as extent of reversible adhesion between material surfaces. The long term goal is to design materials with unprecedented abilities to dynamically direct interactions with its surrounding environment

15. SUBJECT TERMS

oligonucleotides, nuclease-resistant nucleic acid analogs, competitive hybridization, colloidal particles, cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Valeria Milam
UU	UU	UU	UU		19b. TELEPHONE NUMBER 404-894-2845

Report Title

Final Report: Implementing Material Surfaces with an Adhesive Switch

ABSTRACT

The work focuses on implementing material surfaces that present temporal adhesive cues. We are exploring conformational changes in surface-immobilized oligonucleotides to promote an "off-on" switch in adhesion events on the surface of colloidal particles. The overall objective is to optimize oligonucleotide characteristics (e.g. base length, modified nucleotide content, etc.) for chemically-robust macromolecules in order to tailor both the timing as well as extent of reversible adhesion between material surfaces. The long term goal is to design materials with unprecedented abilities to dynamically direct interactions with its surrounding environment.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
02/25/2014	5.00	James O. Hardin, Valeria T. Milam. Correction to Measuring in Situ Primary and Competitive DNA Hybridization Activity on Microspheres, Biomacromolecules, (08 2013): 2961. doi: 10.1021/bm400917a
02/25/2014	6.00	Alberto Fernandez-Nieves, James O. Hardin, Carlos J. Martinez, Valeria T. Milam. Altering Colloidal Surface Functionalization Using DNA Encapsulated Inside Monodisperse Gelatin Microsphere Templates, Langmuir, (05 2013): 5534. doi: 10.1021/la400280x
02/25/2014	7.00	Bryan A. Baker, Gita Mahmoudabadi, Valeria Tohver Milam. Strand displacement in DNA-based materials systems, Soft Matter, (11 2013): 11160. doi: 10.1039/c3sm52157e
04/06/2013	2.00	Ngozi A. Eze, Valeria Tohver Milam. Exploring locked nucleic acids as a bio-inspired materials assembly and disassembly tool, Soft Matter, (02 2013): 2403. doi: 10.1039/c2sm27021h
04/06/2013	4.00	James O. Hardin, Valeria T. Milam. Measuring in Situ Primary and Competitive DNA Hybridization Activity on Microspheres, Biomacromolecules, (03 2013): 0. doi: 10.1021/bm3017466
04/06/2013	3.00	Bryan A. Baker, Gita Mahmoudabadi, Valeria T. Milam. Using double-stranded DNA probes to promote specificity in target capture, Colloids and Surfaces B: Biointerfaces, (02 2013): 884. doi: 10.1016/j.colsurfb.2012.09.020
09/02/2011	1.00	B. A. Baker, V. T. Milam. Hybridization kinetics between immobilized double-stranded DNA probes and targets containing embedded recognition segments, Nucleic Acids Research, (05 2011): 0. doi: 10.1093/nar/gkr293
TOTAL:	**7**	

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals: 5

(c) Presentations

Presentations given 9/2009-2/2014

Five Invited seminars:
1. Clemson University, SC "Programmable release from model drug carriers" (October 20, 2011)
2. Georgia Institute of Technology, Integrated Biosystems Institute, Atlanta, GA "Colloidal Platforms for Nucleic Acid Detection" (February 23, 2011)
3. Georgia Institute of Technology, Institute of Bioengineering and Biosciences, Atlanta, GA "Colloidal Particles as Substrates for Double-Stranded Probes" (March 16, 2010)
4. Washington University, St. Louis, MO "Programming the Isothermal Disassembly of DNA-linked Particles" (January 28, 2010)
5. CCNY, New York, NY "Programming Nanoparticle Release from Colloidal Satellite Assemblies" (November 16, 2009)

Five Invited Conference Presentations
1. V.T. Milam, N. Eze, Composites at Lake Louise Conference, "Modified Oligonucleotides in Reversible Assembly Schemes for Materials," Lake Louise, Alberta, Canada (November 2013)
2. V.T. Milam, J.O. Hardin, A. Fernandez-Nieves, Composites at Lake Louise Conference, "Encapsulation and Release of Active Oligonucleotides for Drug Delivery Applications" Lake Louise, Alberta, Canada (November 2011)
3. V.T. Milam, B.A. Baker, G. Mahmoudabadi 8th International Workshop on Interfaces "Oligonucleotide-modified Colloids for Biosensing Applications" Santiago de Compostela, Spain (June 2011)
4. V.T. Milam, B.A. Baker, C.K. Tison, "Biocolloids for Biomedical and Sensing Applications," 5th Biennial "Molecular Bionics - From Biomineralization to Functional Materials" Symposium, Schloss Ringberg (Rottach-Egern), Germany (October 2010)
5. V.T. Milam, C.K. Tison, J.O. Hardin IV, S.T. Parpart, Composites at Lake Louise Conference, "Programming Nanoparticle Release from DNA-linked Colloidal Satellites," Alberta, Canada (November 2009)

One Invited Workshop Presentation
1. V.T. Milam, 2010 BioInterfaces Workshop, "Employing Double-Stranded Probes on Colloidal Particles for Nucleic Acid Detection" Atlanta, GA (October 2010)

Eight Other Conference Presentations with VT Milam as co-author
(* indicates presentations given by V.T. Milam)
1. V.T. Milam*, N.A. Eze, J.O. Hardin, "Employing Locked Nucleic Acids for Recognition-based Colloidal Assembly & Disassembly" poster presentation at 3rd International Conference on Multifunctional, Hybrid, and Nanomaterials" Sorrento, Italy (March 2013)
2. V.T. Milam*, N.A. Eze, J.O. Hardin, "Employing nucleic acid analogs for recognition-based colloidal assembly & disassembly" 17th Annual Regenerative Medicine, Technologies Enabling Novel Therapies Workshop, Hilton Head Island, SC (March 2013)
3. N. A. Eze, V.T. Milam, 2012 Spring Materials Research Society Meeting, "Exploring locked nucleic acids as a reversible biomaterials assembly tool," San Francisco, CA (April 2012)
4. V.T. Milam*, J.O. Hardin, A. Fernandez-Nieves, 2012 Spring Materials Research Society Meeting "Tunable release of active oligonucleotides from uncrosslinked gelatin microspheres," San Francisco, CA (April 2012)
5. V.T. Milam*, J.O. Hardin, A. Fernandez-Nieves, 2012 American Physical Society Meeting, "Measuring in situ primary and competitive hybridization events on microspheres," Boston, MA (February 2012)
6. J.O. Hardin, A. Fernandez-Nieves, V.T. Milam, poster presentation at 5th Biennial "Molecular Bionics - From Biomineralization to Functional Materials" Symposium "Controlled release of oligonucleotides from a thermosensitive hydrogel," Schloss Ringberg (Rottach-Egern), Germany (October 2010); JOH received partial travel support from the Graduate Conference Funds (GT)
7. V.T. Milam*, C.K. Tison, J.O. Hardin, S.T. Parpart, XIX International Materials Research Congress "Expanding oligonucleotides as programmable assembly and disassembly tools for physiological applications" Cancun, Mexico (August 2010)
8. V.T. Milam*, B.A. Baker, G. Mahmoudabadi, XIX International Materials Research Congress "Nucleic Acid Detection Using Colloidal Substrates for Double-Stranded Probes" Cancun, Mexico (August 2010)

Number of Presentations: 19.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Paper

TOTAL:

Patents Submitted

Patents Awarded

Awards

Honors & Awards to VT Milam - received since September 2009
3M Non-Tenured Faculty Award (2011)
NSF CAREER (2009)

Honors and Awards to graduate students supported through this ARO grant
1. Ngozi A. Eze - Recipient of 2009-2010, 2011-2012 GAANN Fellowship in CD4
2. James A. Hardin - Recipient of 2006-2007, 2008-2009, 2010-2011 GAANN Fellowship in CD4; Currently, Intelligence Community Postdoctoral Fellow at Harvard University
3. Bryan A. Baker - NRC Postdoctoral Fellow at NIST (2011-present)
4. Alex A. Weller - Recipient of 2012-2013 GAANN Fellowship in CD4

Honors & Awards to Undergraduate Researcher:
1. Gita Mahmoudabadi (2009S – 2011S) B.S. in BME with highest honors (May 2011)
Undergraduate Thesis Title: The Effects of Sequence Characteristics on Competitive Hybridization Kinetics Undergraduate Petit Scholar (2009); Pursuing a Ph.D. in Bioengineering at Caltech; awarded a NSF Graduate Fellowship (2011)

Particular Notes on Two Relevant Publications:
1. B.A. Baker, G. Mahmoudabadi, V.T. Milam, "Strand displacement in DNA-based materials systems," Soft Matter, 2013 9 (47) 11160-11172 (Featured as 2013 "Hot Paper" by Soft Matter)
2. N.A. Eze, V.T. Milam, "Exploring locked nucleic acids as a bio-inspired materials assembly and disassembly tool," Soft Matter 2013 9 (8) 2403-2411 (Featured on Cover)

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Ngozi A. Eze	0.40	
James A. Hardin IV	0.40	
Bryan A. Baker	1.00	
Alex Weller	0.40	
FTE Equivalent:	**2.20**	
Total Number:	**4**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Bryan A. Baker	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Valeria Tohver Milam	0.20	
FTE Equivalent:	**0.20**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Gita Mahmoudabadi	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 1.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 1.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 1.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 1.00

Names of Personnel receiving masters degrees

NAME	
Alex Weller	
Total Number:	**1**

Names of personnel receiving PHDs

NAME	
Ngozi A. Eze	
James A. Hardin	
Bryan A. Baker	
Total Number:	**3**

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

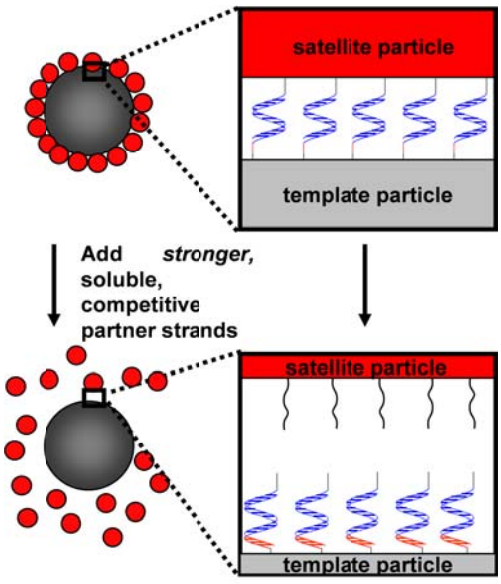
Scientific Progress

Please see Attachment

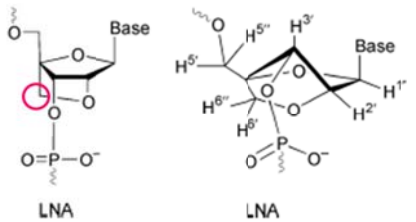
Title: Implementing Material Surfaces an Adhesive Switch
Agreement Number: W911NF-09-1-0479
Author: Valeria Tohver Milam (PI)
Sponsor: ARO
Program Manager: Dr. David Stepp
Final Report Date: 2/26/2014 (covering progress 9/4/2009-9/3/2013)

List of Appendices
Appendix A. Tables of sequences studied.

Statement of Problem Studied
While materials with rich possibilities in "static" structure-function relationships exist, implementing dynamic capabilities on material surfaces provides exciting possibilities for enabling materials with properties that can adjust to a changing environment. While we[1, 2] and others[3-6] have explored DNA as a programmable materials assembly tool, the conventional approach[3-6] to reversing DNA-mediated adhesion events typically entails using elevated temperature conditions to thermally dissociate duplex "bridges" between material surfaces. Another limitation to DNA, particularly in a harsher environment such as the *in vivo* setting, is chemical susceptibility of natural oligonucleotides to, for example, cleavage by nucleases present in many physiological fluids. To address both of these limitations and challenges in the current work, we have successfully employed displacement-based strategies to drive the exchange of one soluble partner strand for another in duplexes. This displacement approach has been used to drive displacement of both immobilized strands (see **Scheme 1**) as well as soluble strands. We have also incorporated nucleic acid analogs called locked nucleic acids (LNA) in sequence to improve their chemical stability in harsh environments (see **Scheme 2**) Furthermore, prior analysis of the *in situ* activity of oligonucleotides on surfaces has typically been limited to planar substrates using techniques such as SPR[7, 8]. To extend *in situ* characterization of colloidal particles, we have adapted a high throughput technique known as flow cytometry in order to quantitatively assess initial binding and displacement activity of both DNA and LNA-based sequences. While much of our work focused on using polystyrene lattices as model particles, we have also demonstrated that oligonucleotides can be temporarily sequestered inside semi-permeable microspheres and then released for subsequent binding activity. Collectively, this body of work present exciting possibilities for employing macromolecular linkers in numerous materials schemes ranging from colloidal assemblies with tunable disassembly kinetics to materials with "on-off" adhesive switches at the surface to,



Scheme 1. Schematic presentation of displacement-based disassembly of oligonucleotide-linked colloidal satellites comprised of a central template particle (e.g. nonfluorescent microsphere) initially surrounded by adherent satellite particles (e.g. fluorescent nanoparticles). The recognition domain or hybridization segment common to both 1° duplexes (promoting assembly in top illustration) and 2° duplexes (promoting disassembly in bottom illustration) is shown in blue. The toehold domain or hybridization segment present only in 2° duplexes is shown in red. Displacement-based strategies have also been employed in this ARO-funded work to replace a soluble (i.e. not immobilized) 1° target with a 2° target.



Scheme 2. LNA nucleotide showing (left) the methylene bridge (highlighted with a pink circle) that "locks" the sugar moiety into (right) a 3′-endo conformation. (Adapted from Jensen *et al. J. Chem. Soc., Perkin Trans.* **2001**, *2*, 1224-1232)

for example, initially camouflage, then reveal a material for a particular time-sensitive function.

Summary of Most Important Results from Published and Unpublished Work

(A) Assessing DNA-LNA mixmer sequences for robust, reversible colloidal assembly schemes

Publication: N. Eze, V.T. Milam, "Exploring locked nucleic acids as a bio-inspired materials assembly and disassembly tool," *Soft Matter* **2013** 9 (8) 2403-2411. (Featured on Cover) This publication demonstrates the ability to incorporate modified oligonucleotides called locked nucleic acids (LNA) into sequences to (i) promote recognition-based colloidal assembly and (ii) recognition-based displacement events to mediate colloidal disassembly at 37 °C following a 24 h incubation with secondary target sequences. Sequences are provided in **Table 1** (see **Appendix A**)

♦ *Flow Cytometry Analysis of Competitive Displacement Activity*

Fig. 1 shows a quantitative comparison of primary mismatched, **L^{3}M11**-, **L^{3}M13**- and **L^{3}M15**-based duplexes remaining hybridized following incubation with various secondary targets for 24 h at room temperature. With the addition of buffer only or noncomplementary **NC14** secondary target, LNA:LNA duplexes retain nearly the same duplex densities indicating negligible thermal dissociation occurs at room temperature. Following incubation with complementary secondary **B15** or **L^{3}B15** targets, however, the duplex densities drop for all shorter mismatched cases. This decrease in the primary duplex density is attributed to competitive displacement of the original hybridization partner by the complementary secondary target. Moreover, as the length of the primary target increases, more primary duplexes remained hybridized in the presence of either **B15** or **L^{3}B15** secondary targets. Thus, despite similar initial primary duplex densities for the **L^{3}M11**, **L^{3}M13**, and **L^{3}M15** cases (see Buffer only case in **Fig. 1**), the mismatched LNA targets do exhibit a sequence length-dependence with respect to displacement activity by both **B15** and **L^{3}B15**. One can infer from these trends in displacement activity that increasing the total number of base-pair matches results in stronger primary duplexes that are less likely to allow for partner exchange. ***Moreover, while quantifying primary hybridization activity is one indicator of relative affinity between oligonucleotide partner strands, differences in competitive displacement activity serve as a better affinity indicator.*** While significant displacement activity by **L^{3}B15** is evident for **L^{3}M11** (reduced from 28,150 to 10,920 oligos/μm^2), and **L^{3}M13** (reduced from 27,400 to 14,610 oligos/μm^2) primary targets**,** the weaker **L^{3}M9** (reduced from 390 to 50 oligos/μm^2) holds better promise for promoting oligonucleotide-mediated assembly as well as complete displacement-based disassembly of colloidal satellites. To validate this sequence choice, competitive displacement activity for select, closely related primary targets (**L^{3}M9** and **L^{3}B9**) were also carried out under conditions mimicking disassembly experiments (i.e., 37 °C conditions; diluted probe coupling step). Under these conditions, the **L^{3}B9** and **L^{3}M9** targets exhibited a 22% and 35% reduction in duplex density, respectively (data not shown, but provided in above publication).

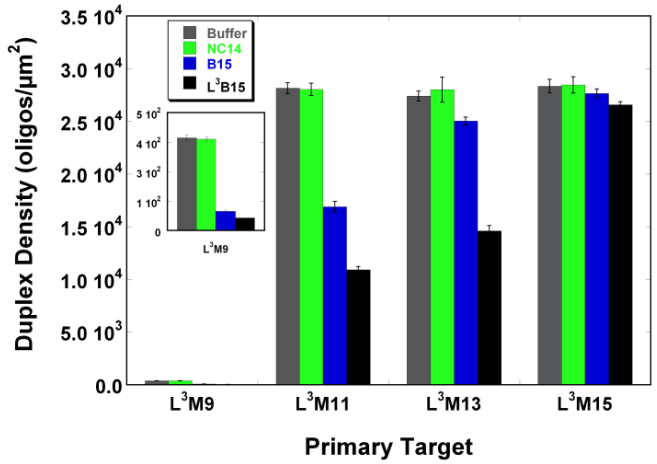


Fig. 1. Surface density of 1° LNA:LNA duplexes remaining following 24 h incubation (followed by routine washes) in the absence (**Buffer**) or presence of secondary DNA (**NC14**, **B15**) or LNA (**L^{3}B15**) target strands at room temperature. Taken from Eze & Milam, *Soft Matter* **2013** 9 (8) 2403-2411.

- *Assembly via Primary Hybridization Events and Disassembly via Competitive Displacement Events*

For assembly and disassembly studies, the immobilized probe concentration was intentionally diluted in order to reduce the number of duplex bridges between colloidal particles in a "colloidal satellite" assembly and enable subsequent disassembly via competitive hybridization (or displacement) events once a secondary target is introduced. Flow cytometry results (not shown) indicated that the initial primary duplex density was reduced ~3-4-fold for **L^{3}A20:L^{3}M9** and **L^{3}A20:B9**-based duplexes. Competitive displacement of these primary hybridization partners was ~95%. **Fig. 2** and **Fig. 3** shows confocal micrographs of **L^{3}A20:*i*L^{3}B9** and **L^{3}A20:*i*L^{3}M9**-linked colloidal satellite assemblies formed at room temperature and then

incubated with noncomplementary secondary targets (**NC14**) or complementary secondary targets (**B15** or **L^{3}B15**) at 37 °C. Although significant thermal dissociation (~35% and 22% for **L^{3}B9** and **L^{3}M9**, respectively) at 37 °C was noted in separate flow cytometry studies (data not shown), the lack of disassembly in the presence of **NC14** secondary target indicates that thermal dissociation alone does not induce nanoparticle release, as shown in **Fig. 2(a)** and **Fig. 3(a)**. While **L^{3}A20:*i*L^{3}B9**-linked assemblies remain fairly intact following incubation with either **L^{3}B15** or **B15** secondary targets present (see Fig. **2(b)** and **2(c)**) extensive disassembly does occur, however, after incubating **L^{3}A20:*i*L^{3}M9**-linked assemblies with the complementary DNA (**B15**) or LNA (**L^{3}B15**) secondary targets, leaving released fluorescent nanoparticles and relatively bare, nonfluorescent probe-functionalized microspheres (not visible in fluorescence micrographs) shown in **Fig. 3(b)–(c)**. ***Collectively, results indicate that LNA-mediated assembly is achieved for perfectly-matched and mismatched LNA-based duplex bridges, but extensive disassembly via competitive displacement events is favored by employing the weaker, mismatched strands as duplex bridges in LNA-linked assemblies.***

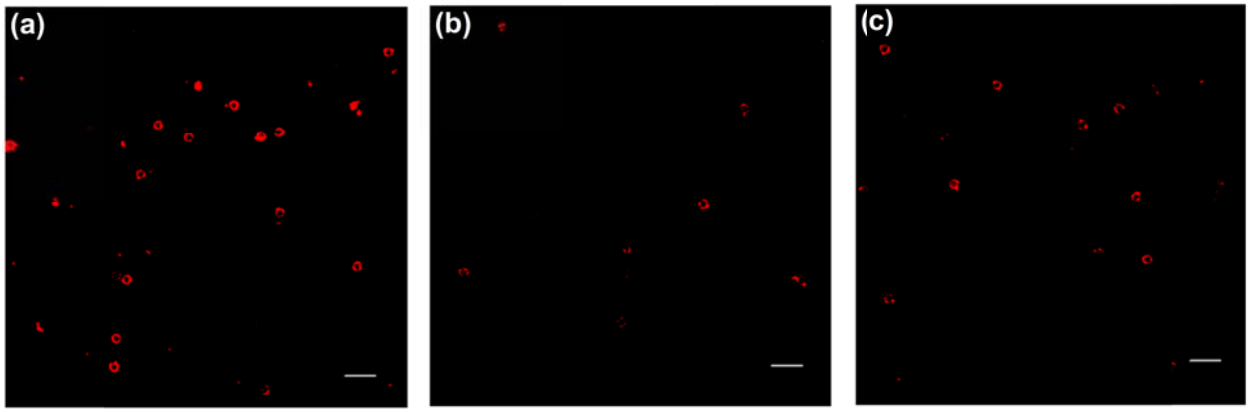


Fig. 2 Suspensions of LNA-linked (**L^{3}A20**:***i*L^{3}B9**) colloidal satellites following a 24 h incubation at 37 °C with (a)

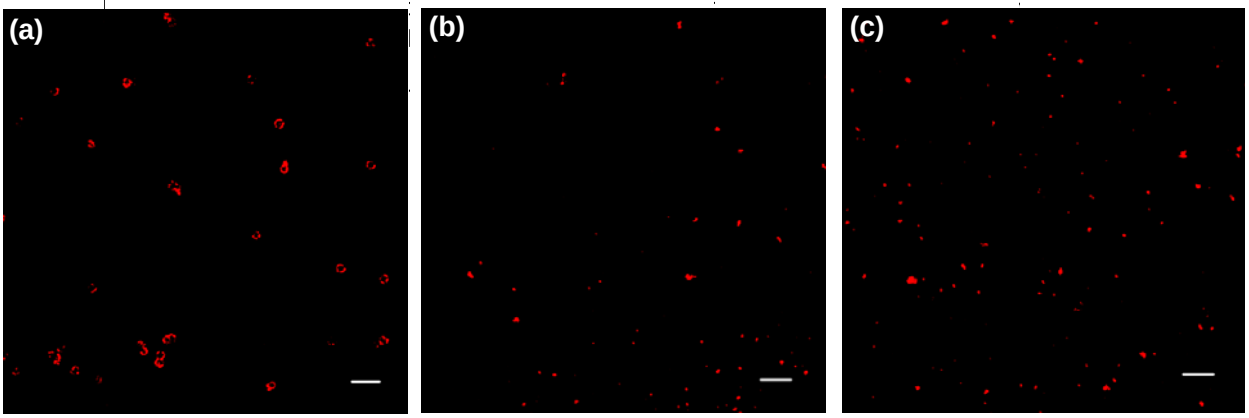


Fig. 3 Suspensions of LNA-linked (**L^{3}A20**: ***i*L^{3}M9**) colloidal satellites following a 24 h incubation at 37 °C with (a) noncomplementary **NC14**, (b) complementary **B15**, or (c) complementary **L^{3}B15** secondary targets. Each micrograph consists of a single focal plane taken in fluorescence-only mode. Scale bars are 5 μm. (Taken from N. Eze, V.T. Milam, *Soft Matter* **2013** 9 (8) 2403-2411).

- *Comparing nuclease resistance of DNA:LNA and LNA:LNA duplexes*

Fig. 4 compares the relative stability of several duplex sequences under various temperature conditions in the absence and presence of DNase I (endonuclease that hydrolytically cleaves double-stranded DNA). Room temperature and 37 °C controls (without DNase I) are included to evaluate thermal dissociation effects. Compared to DNA:LNA duplexes (~65% loss in duplex density) the small differences in duplex density for these two controls indicates that LNA:LNA duplexes (less than ~~20% loss) are more stable. Following incubation with DNAse I (at 37 °C) 99% of the remaining LNA:DNA duplexes are degraded while the LNA:LNA duplex density value remains relatively unchanged. ***These results***

demonstrate that superior thermal and nuclease resistance is conferred on oligonucleotides by substituting ~one-third of DNA nucleotides with LNA nucleotides in hybridization segments – an important consideration in implementing chemically robust macromolecular players.

(B) Measuring in situ hybridization activity of immobilized DNA strands on colloidal carriers

Publication: J.O. Hardin, V.T. Milam, "Measuring in situ primary and competitive DNA hybridization activity on microspheres," *Biomacromolecules* **2013** 14 (4) 986-992. This publication demonstrates the ability to use flow cytometry to perform high throughput *in situ* measurements of primary duplex formation and competitive displacement activity on microspheres. The pure DNA targets are identical in base content to similarly-named LNA-DNA "mixmer" targets in **Table 1** (see **Appendix A**). The experimental approach from these recently published studies have been extended to include LNA nucleotides in either the probe strands immobilized on microsphere surfaces, primary targets, and/or competitive target sequences for select case studies (see **Section (G)**). Although flow cytometry is routinely used by research groups to quantify fluorescently-tagged duplexes on microspheres following conventional wash steps, ***to our knowledge our report is the first to demonstrate adapting this high throughput approach to quantify target binding and displacement events as they occur on microspheres by excluding intermittent wash steps*.**

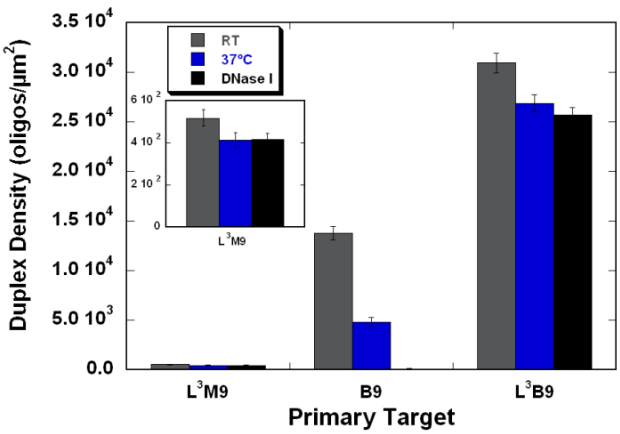


Fig. 4. Primary duplex density remaining between **L³A20** LNA probes and various DNA (**B9**) and **LNA** (**L³M9**, **L³B9**) targets, following incubation for 24 h at room temperature, 37 °C, and 37 °C with DNase I (1 U/mL). Taken from Eze & Milam, *Soft Matter* **2013** 9 (8) 2403-2411.

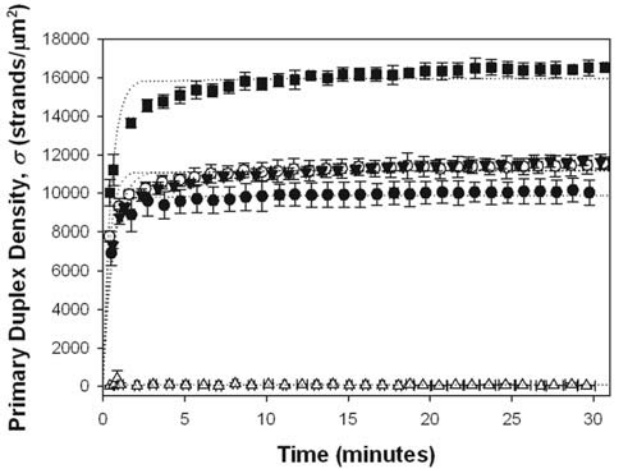


Fig. 5. *In situ* measurements of primary duplex formation between **DNA**(**A20**)-functionalized microspheres and fluorescently-labeled **B15** (solid squares), **M15** (solid triangles), **M13** (open circles), **M11** (solid circles), or **NC14** (open triangles) **DNA** primary targets. Error bars indicating standard deviation for both surface density and time values are shown. Taken from Hardin. & Milam, *Biomacromolecules* **2013** 14 (4) 986-992.

- *Quantifying in situ 1° hybridization events*

To investigate *in situ* primary duplex formation as it occurs between immobilized probes and various targets, suspensions of DNA-functionalized microspheres were interrogated with flow cytometry immediately following introduction to various fluorescently-labeled primary targets. Importantly, these *in situ* measurements were conducted in the absence of any wash steps normally used to remove unassociated or weakly bound target from the vicinity of the microsphere. As shown in **Fig. 5**, all five target cases reach a plateau value in duplex density within 30 min of target introduction indicating that equilibrium was reached within the experimental timeframe. There are, however, negligible differences in the fluorescence intensity of DNA-functionalized microspheres alone and in the presence of noncomplementary **NC14** strands indicating that nonspecific target adsorption to the microsphere surface as well as association between noncomplementary targets and immobilized probes are minimal, even in the absence of any wash steps. ***The nearly identical hybridization rate constants (~0.03-0.04 s^{-1}) for the various targets indicates that the primary hybridization rates are independent of sequence length and fidelity. Moreover, the extent of in situ hybridization is nearly identical for all the mismatched strands, but greater for the perfectly matched strands.***

• *Quantifying in situ competitive displacement*

In situ displacement studies were conducted by directly sampling the suspension at various incubation times with **B15** secondary targets. Since the experimental time frame is only 30 min for *in situ* measurements, the 11 base-long mismatch **M11** DNA target was selected as the primary target since it is readily displaced in separate, posthybridization washing studies (results not shown here, but provided in same publication by Hardin & Milam). Importantly, controls in the absence of complementary target indicated dissociation of this DNA primary target is significant (~40%) over the 30 min experimental timeframe. To account for these thermal dissociation events, release profiles are normalized using these controls in order to discern primary target release events arising from displacement activity. The results of these measurements are shown in **Fig. 6** as fraction (of primary target) displaced over time in the presence of complementary **B15** secondary targets. The resulting values for the observed displacement rate constant, k_2, range from $6x10^{-4}$ to $3x10^{-3}$ s^{-1}, depending on the concentration of secondary targets. Analogous displacement kinetics were evaluated for suspensions that subjected to conventional posthybridization washes (unlike the *in situ* studies). In general, while washing suspensions result in more extensive release of weaker primary hybridization partners, the overall trends in time-dependent displacement activity (e.g. k_2 values, etc.) are similar between *in situ* and postwashing studies. ***Overall, while prior measurements of in situ hybridization activity between immobilized probes and soluble targets have relied on planar material substrates, the current work successfully demonstrates a high throughput analytical approach to monitor hybridization events as they occur on colloidal particles.***

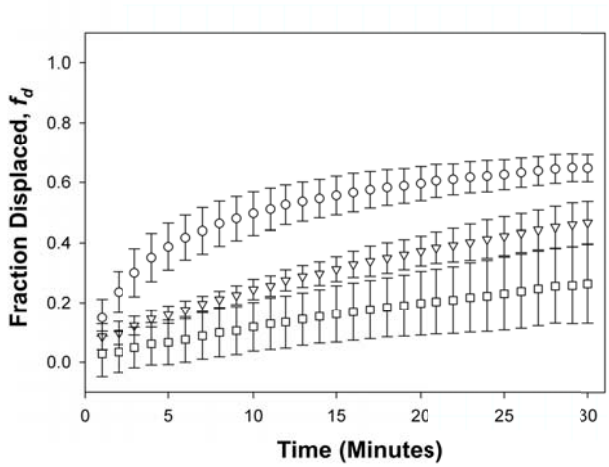


Fig 6. *In situ* measurements of the fraction of primary target, **M11**, displaced from **DNA**(**A20**)-functionalized microspheres by **B15** secondary targets at concentrations of 10 nM **(**squares), 100 nM (triangles), and 1,000 nM (circles). Hardin. & Milam, *Biomacromolecules* **2013** 14 (4) 986-992.

(C) Using competitive displacement activity to promote duplex conversion on colloidal satellites comprised of gelatin microsphere (GMS) cores

Publication: J.O. Hardin, A. Fernandez-Nieves, C.J. Martinez, V.T. Milam, "Altering colloidal surface functionalization using DNA encapsulated inside monodisperse gelatin microsphere templates," *Langmuir* **2013** *29* (18) 5534–5539. Soluble oligonucleotides are typically introduced to bulk solution to promote hybridization activity on DNA-functionalized surfaces. Here, an alternative approach is successfully implemented by encapsulating secondary target strands inside semi-permeable colloidal satellite assemblies, then triggering their release from the satellite "core" for subsequent surface hybridization activity on the adherent

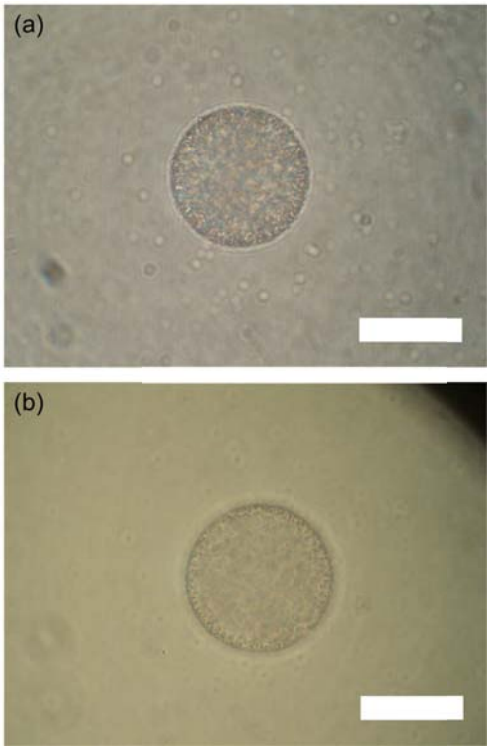


Fig. 7 Phase contrast micrographs of colloidal satellite assembly comprised of a layer of DNA duplex-functionalized microspheres (1.1 μm diameter) adsorbed on a gelatin microsphere (~35 μm diameter) at (a) room temperature and (b) following incubation at 37 ^{o}C overnight. Scale bars represent 25 μm. Taken from Hardin *et al. Langmuir* **2013** *29* (18) 5534–5539

satellite nanoparticles. To prepare DNA-loaded satellite assemblies, uniform gelatin microspheres (GMS) were fabricated using microfluidics, loaded with 15 base-long secondary DNA targets, capped with a polyelectrolyte bilayer, and finally coated with a monolayer of polystyrene microspheres functionalized with short primary duplexes. Once warmed to 37° C, satellite assemblies remain intact while secondary DNA targets are released from the gelatin template to then competitively displace the shorter, original hybridization partners on satellite microspheres. ***This approach involving the encapsulation of hybridization partners inside colloidal matrices shows promise of our capabilities to implement a multi-particle colloidal carrier (e.g. colloidal satellite assembly) that provides its own the release agent (i.e. secondary oligonucleotide targets that are temporarily encapsulated inside colloidal matrix, but later released to competitively displace primary duplex partners) in future work.***

(D) Comparing RNA and DNA as secondary targets

Publication: B.A. Baker, G. Mahmoudabadi, V.T. Milam, "Using Double-Stranded DNA Probes to Promote Specificity in Target Capture," *Colloids and Surfaces B: Biointerfaces* **2013** 102 884-890. In this study we compare the competitive displacement capabilities of 15 base-long, closely related (e.g. differences only in one base), but still distinct RNA (**R15**) or DNA (perfectly-matched **T15**; **T15x3** with near end mismatch; **T15m** with center mismatch) sequences (see **Table 2** in **Appendix A**). The primary hybridization partners was either 13 base-long perfectly matched duplexes (**3P:T13**) or 15 base-long, mismatched duplexes (**5P:T15m**) derived from the Salmonella genome (unlike other studies in **Subsections (A)-(C)** above involving "designer" sequences with no genomic relevance). The displacement of a labeled primary hybridization partner by an unlabeled competitive hybridization partner was measured on microspheres using flow cytometry. Any thermal dissociation was accounted for using control experiments involving the absence of complementary secondary target. Highlights of the study are shown in **Fig. 8**. Intriguingly, the overall trends as well as the total fraction of primary partner strands ultimately displaced for the **5P:T15m** case (see **Figure 8(a)**) remain similar throughout the timeframe of the experiment for all targets considered. In contrast, the extent of displacement in the profiles for the **3P:T13** case (see **Figure 8(b)**) occurs in descending order for **R15**, **T15**, **T15x3**, and **T15m** targets. From these results there is a clear distinction in the timing and extent of competitive displacement activity for the **3P:T13** and **5P:T15m**-based primary duplexes. This ability to "tune" the timing and extent of competitive hybridization activity shows promise towards our ultimate goal of programming the responsiveness of "stealth coatings" on material surfaces using oligonucleotides. While DNA and RNA are not as chemically robust as LNA, our past and ongoing work indicates similar abilities to tune the responsiveness of LNA-based sequences.

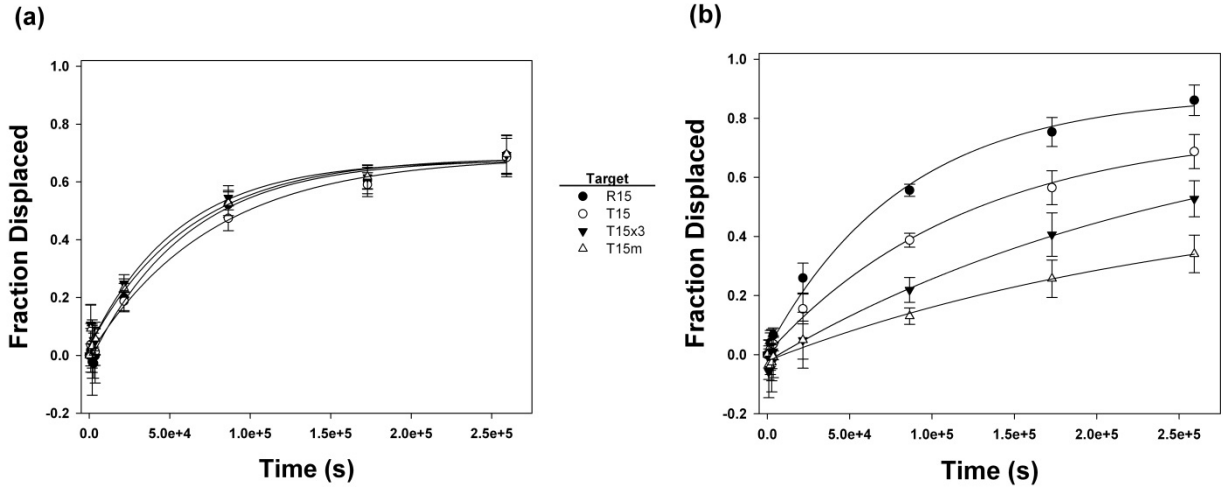


Fig. 8 Fraction of reporter strands displaced as a function of incubation time with various RNA and DNA targets from (a) **5P:T15m** and (b) **3P:T13** dsProbes. Each data point represents the average of three separate measurements with error bars corresponding to the standard deviation about this average.

(E) Employing competitive displacement events in materials systems

Publication: B.A. Baker, G. Mahmoudabadi, V.T. Milam "Strand displacement in DNA-based materials systems" *Soft Matter* **2013** 9 (47) 11160-11172. (Highlighted as 2013 "Hot Paper" by *Soft Matter*). While

primary hybridization between single-stranded DNA oligonucleotides has been well-studied in the literature and successfully employed in numerous materials assembly schemes, this review article highlights a related, but distinct activity involving the exchange of one partner strand for a second, often strong binding partner in a duplex. The review article highlights displacement-based strategies in scenarios ranging from DNA lattices with expandable dimensions to colloidal assemblies in which particles can rearrange or redisperse.

(F) Investigating how the spatial location of the "recognition domain" in a secondary target affects displacement kinetics.

Publication: B.A. Baker, G. Mahmoudabadi, V.T. Milam "Hybridization kinetics between immobilized double-stranded DNA probes and targets containing embedded recognition segments," *Nucleic Acids Research* **2011** 39 (15) e99. The time-dependent strand displacement activity of several targets with double-stranded DNA probes (dsProbes) of varying affinity was studied using sequences shown in **Table 3** (see **Appendix A**). Here, the relative affinity of various dsProbes is altered through choices in hybridization length (11-15 bases) and the selective inclusion of center mismatches in the duplexes. While the dsProbes are immobilized on microspheres, the soluble, 15 base-long complementary sequence is presented either alone as a short target strand or as a recognition segment embedded within a longer target strand. Compared to the short target, strand displacement activity of the longer targets is slower, but still successful. Additionally, the longer targets exhibit modest differences in the observed displacement rates, depending on the location of recognition segment within the long target. Overall, this study demonstrates that the kinetics of strand displacement activity can be tuned through dsProbe sequence design parameters and is only modestly affected by the location of the complementary segment within a longer target strand.

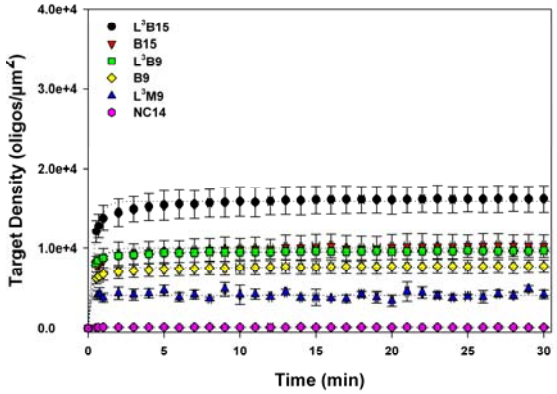


Fig. 9 *In situ* measurements of binding activity between **DNA**-functionalized microspheres and soluble, fluorescently labeled noncomplementary DNA targets (**NC14**); complementary DNA (**B9**, **B15**); or LNA (**L³M9**, **L³B9**, **L³B15**) targets. Dotted lines represent curve fits to the following equation: $\sigma = \sigma_\infty(1 - \exp(-k_1 t))$ where σ is the time-dependent duplex density, σ_∞ corresponds to the σ value at the 30 min time point, k_1 is the duplex association rate constant, and t is time. Error bars indicating standard deviation for duplex density and time values for the average of three suspension samples are shown.

(G) Measuring in situ primary hybridization activity and displacement activity between DNA probes and LNA-DNA mixmer targets.

Representative results shown in **Fig. 9** and **Fig. 10** from recent unpublished studies demonstrate our abilities to quantify binding and release events as they occur between DNA-functionalized microspheres and various DNA and LNA-based targets to directly measure rate constants as well as the extent of oligonucleotide binding activity on microspheres using sequences shown in **Table 1** (see Appendix). Notably, all data sets include a noncomplementary target sequence to assess any nonspecific binding of target to probe-functionalized microspheres (shown in **Fig. 9**) as well as any spontaneous probe:target duplex dissociation (and thereby convert release profiles

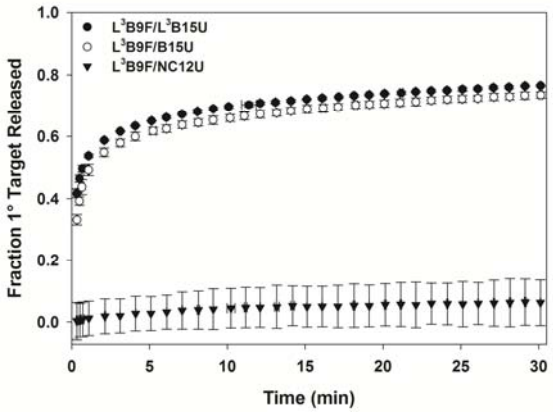


Fig. 10 Measurements of the *in situ* release of 9 base-long LNA targets (**L³B9F**) from DNA **A20**-functionalized microspheres by unlabeled ("**U**") 15 base-long LNA (**L³B15U)** or DNA (**B15U**) secondary targets. Controls with the noncomplementary DNA target (**NC12U**) were used to determine the extent of any duplex dissociation.

shown in **Fig. 10** into displacement profiles not shown). These noncomplementary target controls are essential to include in our kinetic analysis of hybridization and displacement activity. Nearly identical values for the primary duplex association rate constants, k_1, of ~10^{-2} s^{-1} are derived from fits of the duplex formation profiles shown in **Fig. 9** and demonstrate that the primary hybridization rate constants between DNA and various DNA and LNA-DNA targets are independent of LNA content and sequence base length for the sequences studied. Using the noncomplementary target case to account for any thermal dissociation, these release profiles in **Fig. 9** were converted into displacement profiles (not shown for this data set, but see **Fig. 6** for examples of displacement profiles). Similar observed displacement rate constants, k_d, (0.03 s^{-1}) derived from *displacement* profiles for DNA-functionalized microspheres indicate similar displacement capabilities of a 9-base-long LNA-DNA mixmer primary target by either 15 base-long DNA or LNA-based competitive targets.

Two manuscripts are currently in preparation to report these most recent results.

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Appendix A

Table 1. List of oligonucleotide function, nomenclature, and sequence. Note: For simplicity the term "LNA" is used throughout this report to refer to an LNA-DNA mixmer sequence.

Function	Nomenclature	
immobilized DNA probe	**A20 =**	3'–TAGTCGGCGTTAGGTTTTTT–5'
immobilized LNA probe	**L^{3}A20 =**	3'–TA^{L}GTC^{L}GGC^{L}GTT^{L}AGG^{L}TTTTTT–5'
soluble DNA or LNA 1° targets	**B9 =**	5'–ATCAGCCGC–3'
	L^{3}B9 =	5'–AT^{L}CAG^{L}CCG^{L}C–3'
	B11=	3´-AACGCCGACTA-5
	B13=	3´-CTAACGCCGACTA-5´
	B15=	3´-ACCTAACGCCGACTA-5
	M9 =	5'–ATCACCCGC –3'
	M11 =	5'–ATCAGGCGCAA–3'
	M13 =	5'–ATCAGCGGCAATC–3'
	M15 =	5'–ATCAGCCCCAATCCA–3'
	L^{3}M9 =	5'–AT^{L}CAC^{L}CCG^{L}C–3'
	L^{3}M11 =	5'–AT^{L}CAG^{L}GCG^{L}CAA^L–3'
	L^{3}M13 =	5'–AT^{L}CAG^{L}CGG^{L}CAA^{L}TC–3'
	L^{3}M15 =	5'–AT^{L}CAG^{L}CCC^{L}CAA^{L}TCC^{L}A–3'
immobilized LNA-DNA mixmer 1° targets	***i*L^{3}B9 =**	5'–TTTTTTTTTTTTAT^{L}CAG^{L}CCG^{L}C–3'
	***i*L^{3}M9 =**	5'–TTTTTTTTTTTTAT^{L}CAC^{L}CCG^{L}C–3'
soluble DNA 2° target	**B15 =**	5'–ATCAGCCGCAATCCA–3'
soluble LNA 2° target	**L^{3}B15 =**	5'–AT^{L}CAG^{L}CCG^{L}CAA^{L}TCC^{L}A–3'
noncomplementary targets	**NC14 =**	5'–GGATTGCGGCTGAT–3'
	NC12 =	5′-TAGTCGGCGTTA- 3′

[a]Superscript "3" in sequence nomenclature indicates LNA present at every third residue. Superscript "L" after base in a sequence indicates a LNA residue. Underlined base indicates a mismatch.

Table 2. List of Salmonella-based dsProbe and target sequences used in flow cytometry studies between the immobilized strands (**3P** or **5P**) and either the reporter or target strands. The immobilized sequence of each dsProbe contains an amine group on either the 3´ (**3P**) or 5´ (**5P**) end. The reporter sequence of each dsProbe is labeled with a fluorescein derivative (FAM) or (T Fluor) on either the 3´ (**T13**) or 5´ (**T15m**) end. Underlined base indicates a mismatch between the immobilized strand and either the reporter strand or the target strand. Red lettering indicates the single-stranded toehold segment present in **3P:T13** dsProbes.

Nomenclature	Sequence
3P:T13	3' - Amine-TTT TTT ACT ATC ACA CTG C**TC** - 5' 5' - TGA TAG TGT GAC G (FAM)- 3'
5P:T15m	5' - Amine-(12 carbon) CTC GTC ACA CTA TCA - 3' 3' - GAG CAG TCT GAT AGT (T Fluor)- 5'
Nomenclature	**Sequence**
T15	3' - GAG CAG TGT GAT AGT - 5'
T15m	3' - GAG CAG TCT GAT AGT - 5'
T15x3	3' - GAC CAG TGT GAT AGT - 5'
R15	3' - GAG CAG UGU GAU AGU - 5'

Table 3. List of double-stranded DNA probe (dsProbe) sequences used in flow cytometry studies. The top strand in each duplex is immobilized to a microsphere via the amine terminus. The notation "(T Fluor)" in each reporter strand corresponds to the fluorescein-modified thymine that is not intended to participate in hybridization. Single-stranded bases in dsProbes are highlighted in red and any center mismatches are underlined in the reporter strand.

dsProbe Nomenclature	Sequence
P15:NC-18	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – (Fluor T)TT TTT TTT TTT TTT TTT – 5′
P15:T11	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – AG TGT GAT AGT (T Fluor) – 5′
P15:T13	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – G CAG TGT GAT AGT (T Fluor) – 5′
P15:T15	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – GAG CAG TGT GAT AGT (T Fluor) – 5′
P15:T11m	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – AG TGT CAT AGT (T Fluor) – 5′
P15:T13m	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – G CAG TGA GAT AGT (T Fluor) – 5′
P15:T15m	5′ – Amine (12 carbon) CTC GTC ACA CTA TCA – 3′ 3′ – GAG CAG TCT GAT AGT (T Fluor) – 5′